Development of a Plate Assay for the Quantification of ssDNA Micelle Internalization

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Abstract

In this work, the development of a plate assay capable of quantifying the internalization of fluorescently-labeled, single stranded (ssDNA) micelles in murine glioblastoma (GL261) cells was explored. Studies into the internalization pathway of targeted and non-targeted nanoparticles such as carbon nanotubes, TAT peptides, and nanohydrogels have traditionally been carried out using flow cytometry[1,2,3,5]. In these experiments, cells are incubated with the fluorescently-labeled nanoparticles of interest following the blocking of an independent endocytic pathway, and the resulting effect on cell fluorescence, a marker of internalization, is quantified. A plate assay is a useful, higher throughput tool for quantifying cell fluorescence that could compete with flow cytometry as the preferred analytical method for internalization experiments involving the ssDNA nanoparticles under investigation in the Kokkoli Lab. Here, the preparation steps of this assay were based on a published protocol used to explore the mechanism of internalization of various receptor-targeted transfection agents in colon cancer cells using flow cytometry[5]. The data in Figure 2 A-C. represents checkpoints in the development process, with the conditions used to produce the data in Figure 2 D. constituting the current design of the assay moving forward.

Methods

Nanoparticle Synthesis

The oligonucleotide REV 10nt G5 with a 3' amino-modifier (5'-CTCTTGGGGG-AmMo-3') was used for this work, in addition to a modified variant with a HEX fluorophore attached to the 5'-terminus. A published protocol was followed to attach the hydrophobic tail to the oligo, thereby forming a self-assembling amphiphilic molecule[4]. The unreacted ssDNA was separated from the ssDNA-amphiphiles using reverse-phase high performance liquid chromatography (HPLC), and the HPLC spectra is shown in Figure 1.

Cell Culture and Plate Assays

GL261 cells were cultured in TCT T75 cell culture flasks at 37°C and 5% CO_2 using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/V) fetal bovine serum (FBS). Cells were passaged upon reaching 80% confluence using TrypLE Express cell dissociation agent.

Researcher 1 (Michael Harris) and Researcher 2 (Tsvetelina Baryakova), performed the internalization experiments concurrently in order to affirm repeatability of the assay. The preparation steps are a scaled-down variant of a protocol used to determine the internalization pathway of polyplexes, lipoplexes, and stealth lysosomes in α5β1 Integrin Bearing DLD-1 cells[5].

Approximately 10,000 GL261 cells/well were added to a 96-well plate and allowed to grow in 150 μL of DMEM for 24h. After 24h, the media in the wells was replenished. In the first round of development (Figure 2 A.), 0.75 μL of 1 mg/mL filipin III in methanol, a known inhibitor of the caveolae-dependent mechanism of endocytosis in cells, was added in a fraction of the wells in addition to micelles at a final concentration of 12.5 μM per well. The remainder of the wells received only the nanoparticles at the same concentration. All wells were then incubated for 2h, and then rinsed once using 150 μL of phosphate-buffered saline (PBS). The PBS was replenished in the wells prior to imaging. To gauge the effect of the filipin III on nanoparticle uptake, each well

was imaged at a single point at an excitation wavelength of 530 nm and emission wavelength of 560 nm. All data given is taken in triplicate and corrected to account for the naked fluorescence of cells. In the second round (Figure 2 B.), cells were pre-incubated for 30 minutes with or without the addition of the filipin III, and incubated for 1h following the addition of the nanoparticles. In addition, the absorbance reading of the wells was taken using an area-scan that composited 25 individual data points to give a mean reading in relative fluorescence units (RFU), and this was done for all rounds moving forward. In the third round (Figure 2 C.), a second negative control (cells + filipin III) was employed to determine the fluorescence of the filipin III, if any. In the fourth round (Figure 2 D.), a second wash with PBS was performed prior to imaging in all wells. Additionally, the effect of replenishing the media prior to the addition of the nanoparticles was studied.

Results and Observations

The data in Figure 2 A. is not analyzed further since the failure to pre-incubate with filipin III prior to the addition of the nanoparticles deviates from the previously-established protocol being followed. From the data in Figure 2 B., it can be seen that cell death likely occurred for Researcher 2's experiments, due to the absence of any fluorescence in the wells. Moreover, Researcher 1's data between Figure 2 A. and B. differ, affirming the effect of either pre-incubating with filipin III, performing an area scan, or both.

In Figure 2 C., it can be seen that there is better agreement between the experimental data of Researchers 1 and 2. To better characterize the cell-micelle interaction moving forward (i.e. distinguish the loose binding of micelles to the cell surface from internalization), cells were washed twice with PBS instead of once prior to imaging. The effect of an intermediate wash step with PBS prior to the addition of the nanoparticles was also independently studied, as the interaction of the filipin III with the ssDNA was hypothesized to potentially affect binding, internalization, or both, but this ultimately had no impact on the fluorescence.

As seen in Figure 2 D., the inclusion of the second, final wash step drastically changed the trends in the data from previous rounds of experimentation, showing a decrease in micelle internalization for both researchers. This is hypothesized to be because this wash removed the nanoparticles that were loosely bound to the cell surface but not internalized, which may have previously been giving erroneously inflated fluorescence readings in the wells. The preliminary data shown here also demonstrates an additional decrease in internalization for the micelles following the addition of filipin III. This would suggest that the internalization pathway of ssDNA micelles is in part caveolae-dependent; however, the protocol is still under development at this time and more independent measurements need to be performed, thus making the data collected inadmissible until its repeatability is confirmed and uncertainty is removed.

Reflection of Objectives and Conclusion

The initial statement of purpose for this work detailed performing internalization experiments with FKN-S2 (fractalkine-targeting) micelles in MCA-38.FKN cells using flow cytometry by the independent blocking of three pathways (clathrin-dependent endocytosis, caveolae-dependent endocytosis, and micropinocytosis) and gauging the resulting effect on cell fluorescence, a marker for cellular uptake. The project goals shifted towards the development of a high-throughput plate assay using non-targeted ssDNA micelles with GL261 cells, which is potentially adaptable for the quantification of a wider variety of characteristics in fluorescent nanoparticles. This assay shows promise as a useful tool to expedite future internalization experiments.

Personal Reflection

My experience through the Undergraduate Research Opportunities Program has been positive; through it, I was able to receive the tools and support necessary to improve upon the various techniques required for my project. These include the synthesis of ssDNA-amphiphiles, cell culture, HPLC/FPLC, protocol development, plate assays, and more. In addition, my work gave me a better insight into the nature of biological research and a deeper appreciation for the fields of DNA nanotechnology, drug delivery, and academic research in general.

Figures

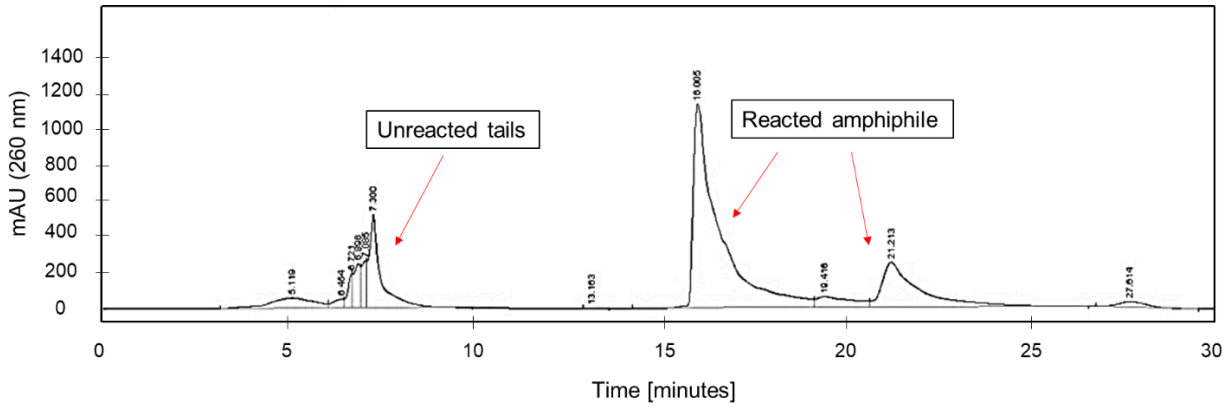

Figure 1| Elution Times and Peaks for HPLC. As shown, the unreacted tails (hydrophobic) eluted between 3-10 minutes, and the reacted amphiphile eluted after 15 minutes.

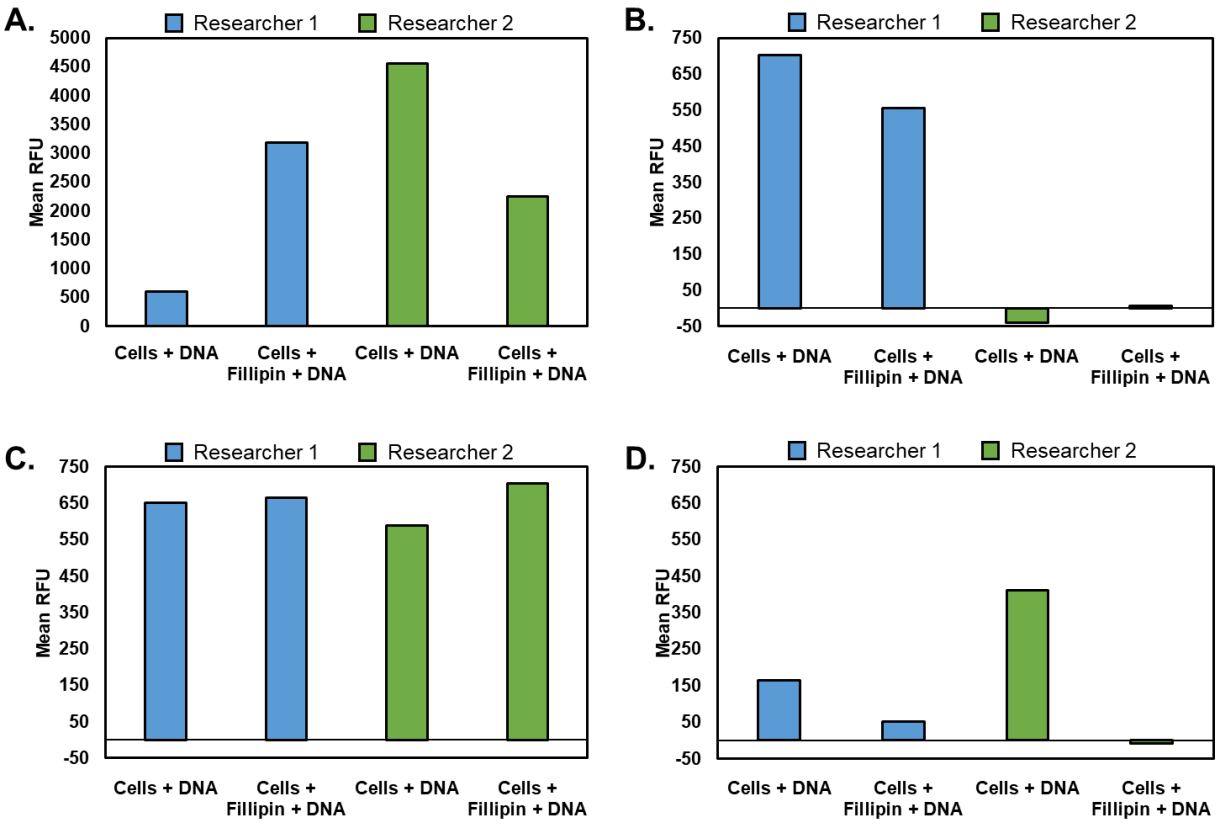

Figure 2 | Fluorescence Data Collected During Development of Plate Assay Protocol. Data is represented as the average of three points in a single set, excluding statistical outliers as identified by a one-tailed Grubbs' test at the 95% confidence interval. **A.** Single point data collection with the concurrent incubation of filipin III and nanoparticles. **B.** Area scan with pre-incubation of filipin III prior to addition of nanoparticles. **C.** Inclusion of a 'cells + filipin III' negative control (no significant effect). **D.** Addition of second wash step with PBS prior to imaging.

References

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